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Properties of a Hydrogen-Inhibited Mutant of *Desulfovibrio desulfuricans* ATCC 27774†

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A mutant of *Desulfovibrio desulfuricans* ATCC 27774 has been obtained which is incapable of sulfate respiration with molecular hydrogen but which grows normally on lactate plus sulfate under argon. Growth characteristics of the mutant suggest that the defect is involved in electron transfer to sulfate or nitrate but not thiosulfate.

Constitutive hydrogenase among species of *Desulfovibrio* may be an adaptation to growth in ecosystems where trace levels of H_2 are present (1, 3). Alternatively, the enzyme may play a role in venting of excess reductant or in the recycling of H_2 (5–7). To clarify the role of hydrogen metabolism during heterotrophic growth and to test the hypothesis of H_2 cycling (6), hydrogenase-negative mutants of a nitrate- or sulfate-respiring organism, *Desulfovibrio desulfuricans* ATCC 27774, were sought. During this search H_2-inhibited mutants were isolated. In this report the properties of one such mutant, designated MO200, were examined. The strain grows normally on lactate plus sulfate under argon or on H_2 plus thiosulfate but is incapable of sulfate reduction with H_2.

D. desulfuricans ATCC 27774 was maintained on medium containing lactate plus sulfate (LS medium) of the following composition: NH_4Cl, 80 mM; KH_2PO_4, 3.6 mM; $MgCl_2 \cdot 6H_2O$, 1 mM; $CaCl_2 \cdot 2H_2O$, 0.7 mM; Na_2SO_4, 30 mM; sodium DL-lactate, 60% (wt/vol) syrup (Sigma Chemical Co.), 60 mM; HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.0), 50 mM; $Na_2S \cdot 9H_2O$, 0.5 mM; cysteine hydrochloride, 0.5 mM; yeast extract (Difco Laboratories), 1 g per liter; and 10 ml of mineral solution 1 per liter (2). Sodium pyruvate, ethanol, choline chloride, or glycerol was substituted at 60 mM each for sodium lactate. $Na_2S_2O_3$ was substituted for Na_2SO_4 at 30 mM. For growth with H_2-CO_2 (80:20), sodium acetate and $NaHCO_3$ were substituted at 22 mM each for sodium lactate.

Growth was measured as an increase in optical density at 660 nm in anaerobic tubes (Bellco) with a Bausch & Lomb Spectronic 70.

Hydrogen-inhibited mutants of strain ATCC 27774 were generated by mutagenesis of wild-type cultures with a GT-8 germicidal lamp followed by 24 h of growth on LS medium. Mutant enrichment consisted of three cycles of alternating lactate-dependent growth (LS medium) with growth under H_2-CO_2 on acetate-bicarbonate medium containing 20 μg of ampicillin per ml. The final LS culture was plated onto LS medium (1.6% [wt/vol] agar) under argon at 31°C. To test for the ability to grow on H_2-CO_2, individual colonies from these plates were transferred by sterile toothpick to acetate-bicarbonate plates under H_2-CO_2. Colonies showing no H_2-dependent growth were replated on LS medium and retested on acetate-bicarbonate medium to confirm the phenotype. Of four mutants isolated, MO200 was selected for further study.

Benzy viologen-, sulfate-, or thiosulfate-dependent H_2 uptake was measured manometrically in Warburg flasks containing the following: Na_2SO_4 or $Na_2S_2O_3$, 30 μmol; or benzyl viologen, 350 μmol; HEPES buffer, 50 μmol; whole cells, 0.5 to 1.0 mg of protein in 1 ml of total volume. H_2 evolution was determined manometrically in Warburg flasks containing the following: sodium pyruvate, 80 μmol; HEPES buffer (pH 7), 50 μmol; whole cell protein, 5 mg in 1 ml of total volume. The center well contained 0.2 ml of 0.2 M NaOH to absorb CO_2. Whole cell protein was determined by the Lowry et al. method (4) after digestion of the samples with 0.2 M NaOH for 1 min at 100°C.

MO200 was isolated as a mutant of *D. desulfuricans* ATCC 27774 unable to grow on H_2 plus sulfate. This phenotype could arise from the inability to either oxidize H_2 or reduce sulfate. These two possibilities were investigated by testing growth with various substrates (Table 1, Fig. 1). Both the mutant and wild-type strains were tested for the

TABLE 1. Maximal cell densities attained by MO200 or wild-type strains on various substrates in the presence or absence of H_2

Substrate[a]	Optical density at 660 nm[b]			
	Wild type		MO200	
	Ar	H_2[c]	Ar	H_2
$H_2 + SO_4^{2-}$		0.45		<0.02
$H_2 + S_2O_3^{2-}$		0.30		0.50
$H_2 + NO_3^-$		0.60		0.05
Pyruvate + SO_4^{2-}	0.55	0.95	0.78	0.10
Pyruvate + $S_2O_3^{2-}$	0.98	0.75	0.95	0.80
Lactate + SO_4^{2-}	0.40	0.50	0.40	<0.02
Lactate + NO_3^-	0.65	0.80	0.05	0.06
Choline + SO_4^{2-}	0.50	0.80	0.40	0.40
Ethanol + SO_4^{2-}	0.06	0.60	0.03	0.07
Glycerol + SO_4^{2-}	0.04	0.70	0.04	0.08

[a] Electron donors were 60 mM and electron acceptors were 30 mM.
[b] Maximal optical density changes after 48 h of growth. Values are representative of three determinations.
[c] Cultures were shaken under 100% H_2.

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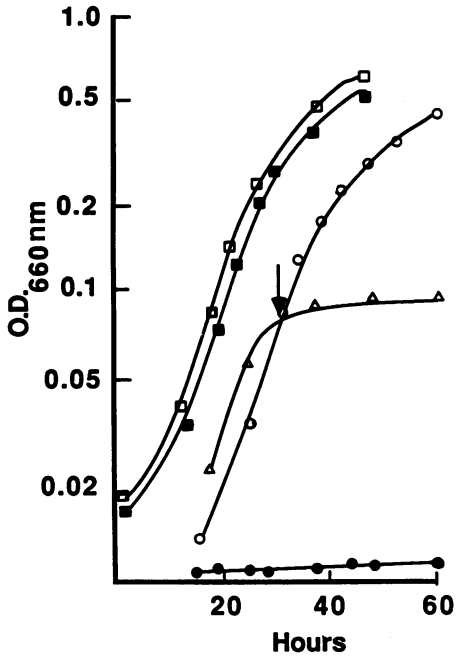

FIG. 1. Growth of wild-type or MO200 strains on sodium lactate plus sulfate in the presence of H_2. Symbols: ■, wild type under argon; □, wild type under H_2; ○, MO200 under argon; ●, MO200 under H_2; △, MO200 initially under argon, which was replaced by 100% H_2 where indicated by the arrow.

effect of H_2 on heterotrophic growth with the alcohols ethanol and glycerol, the organic acids lactate and pyruvate, and the amine choline. H_2 generally stimulated growth of the wild type; H_2 was obligatory for growth on alcohols. H_2 inhibited growth of the mutant with acids or alcohols when sulfate was the electron acceptor. Surprisingly, growth of the mutant with choline plus sulfate was relatively unaffected by H_2, whereas the wild-type growth was stimulated. H_2 was apparently utilized by both strains growing on choline plus sulfate as evidenced by the considerable negative pressure developed. H_2 did not inhibit growth of the mutant when thiosulfate served as the electron acceptor with pyruvate as the electron donor. The mutant grew poorly with lactate and nitrate under argon or H_2 and did not grow at all on H_2 plus nitrate.

When growth occurred, generation times were between 5 and 8 h for both the mutant and the wild type. Growth on pyruvate plus sulfate could be due to growth by pyruvate fermentation, where normal doubling times are on the order of 25 h. Growth of MO200 on lactate plus sulfate was most sensitive to H_2 inhibition. Replacement of the argon headspace gas with 100% H_2 during midlogarithmic growth resulted in immediate cessation of growth (Fig. 1).

The mutant could utilize H_2 for thiosulfate reduction but not sulfate reduction. This observation was substantiated in manometric assays of H_2 uptake with resting cells of the wild type or MO200 grown on pyruvate plus thiosulfate. Rates of benzyl viologen-linked H_2 uptake were comparable for the mutant and wild type at 5 and 9 μmol of H_2 min^{-1} per mg of protein, respectively, indicating that hydrogenase was present in the mutant. Thiosulfate-dependent H_2 uptake rates were similar at 0.4 and 0.5 μmol of H_2 min^{-1} per mg of protein for the mutant and the wild type, respectively. In contrast, sulfate-dependent H_2 uptake rates were eightfold lower in the MO200 suspension (0.06 versus 0.49 μmol of H_2 min^{-1} per mg of protein). Nitrate-dependent hydrogen uptake could not be measured in MO200 due to the inability of this strain to grow on nitrate to any appreciable extent. These respiratory activities reflect the growth capabilities and support the existence of a block in electron transfer from H_2 to sulfate. To test for a block in H_2 evolution from pyruvate, mutant and wild-type resting cell suspensions were manometrically assayed for their ability to evolve H_2. Comparable rates of H_2 evolution of 0.6 to 1 μmol of H_2 h^{-1} per mg of protein were found for both cell types indicating that the block was either not rate limiting for or not involved in pyruvate fermentation.

The data suggest that all essential electron transport components are available for growth on lactate plus sulfate but not on lactate plus nitrate and that exposure to H_2 causes a further change resulting in an inability to grow on sulfate. Therefore the nitrate and sulfate respiratory pathways may share the affected component. Second, since thiosulfate reduction is unaffected under all conditions the affected component is not common to thiosulfate and nitrate reduction. The most likely explanation for the MO200 phenotype is that an essential electron transfer step has been altered such that nitrate reduction is blocked, and in the presence of H_2 electron flow to the activated species of sulfate, adenosine 5′-phosphosulfate, is prevented. The lack of H_2 inhibition for sulfate-dependent growth with choline remains incongruous and may indicate an additional pathway of electron transfer which is unaltered in the mutant. MO200 or the wild type will grow very slowly by choline fermentation in the absence of sulfate; however these growth rates and yields are insufficient to account for the growth observed for MO200 on choline plus sulfate under H_2.

Odom and Peck proposed that H_2 was an obligatory intermediate in the lactate-sulfate respiration via an H_2 cycling mechanism (6). MO200 (i) respires with sulfate under argon, but (ii) it is incapable of growth on H_2 plus sulfate, and (iii) its heterotrophic growth is inhibited by H_2. These facts argue against H_2 being an intermediate in the lactate-to-sulfate respiration. Hydrogenase-negative mutants should contribute substantially to the evaluation of hydrogen metabolism in *Desulfovibrio* species. Assuming that the H_2 inhibition seen in MO200 is mediated by hydrogenase, mutants lacking hydrogenase activity may be found among the revertants of MO200 and are being sought. We anticipate that the isolation of other types of electron-transfer mutants will provide insights into the physiological roles of novel redox proteins which have been discovered in the sulfate-reducing bacteria (7).

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